An Artificial Lipoprotein Carrier System for Pharmaceutical Use

Field of the Invention

The present invention relates to novel carriers for bioactive agents which mimic native lipoprotein, preferably, low density lipoprotein.

Related Applications

This application claims the benefit of priority from provisional patent application serial number 60/234,141, filed September 21, 2000.

Background of the Invention

One of the prerequisites for the action of a drug generally is its ability to penetrate lipid cell membranes. But in order to do this the drug must generally act through its undissociated, lipid soluble part. This chemistry, however, conflicts with the chemistry associated with drug dissolution and its ability to be administered orally or even parenterally, as a drug has to be dissolved in gastric juices in the case of oral administration or a physiological vehicle in the case of parenteral administration, which vehicle normally is an isotonic aqueous solution. Thus, many drugs cannot be administered orally or parenterally unless the drugs can be modified chemically to provide greater dissolution for administration.

Many pharmaceutically active agents now in common use often require formulation compromises in order to prepare the marketed product. In the administration of pharmacologically active agents, it has generally been necessary to use water-soluble agents or to transform the agents into a water-soluble form, so that dissolution properties can be obtained which are appropriate for administration. Thus, many parenteral compositions must be prepared using the salt of the parent compound and an excessive pH. The use of the agents in a water-soluble form, however, has often had several disadvantages. For instance, the excessive pH required for aqueous solutions may often cause side effects. Also, it may sometimes be difficult to attain a desired effect, as the solutions may not be tolerated by the patient.

The instability of many useful drugs and other useful medical compositions poses other formulation problems. At present, emulsions, microemulsions and liposomes constitute the principal approaches to these problems. While such dosage forms are an advance over older forms, they are often associated with erratic bioavailability and instability of their own.

Objects of the Invention

It is an object of the present invention to provide novel formulations for the delivery of bioactive agents.

It is an additional object of the invention to provide novel vehicles which may readily deliver lipophilic (hydrophobic) bioactive agents to a patient without the need to reformulate the agents into more water soluble forms.

It is still another object of the invention to provide novel formulations which mimic lipoproteins such that water-insoluble bioactive agents may be solubilized and delivered to the patient through the LDL pathway.

It is yet a further object of the invention to provide formulations which further comprise proteins or polymers which allow for targeting of the formulations to specific organs or tissues within the patient.

It is still another object of the invention to provide methods for enhancing the bioavailability of bioactive agents using compositions according to the present invention.

One or more of these and/or other objects of the present invention may be readily gleaned from a description of the invention which follows.

Brief Summary of the Invention

The present invention relates to novel carriers for bioactive agents, preferably, hydrophobic drugs for enhanced delivery of the bioactive agents to patient. Compositions according to the present invention comprise a microemulsion which contains a bioactive agent, said microemulsion comprising a lipid core (such as a triglyceride) which is preferably

neutral in pH, stabilized by a monolayer of an amphipathic lipid, preferably a phospholipid and at least one bioactive agent which, depending upon the chemical characteristics of the bioactive agent, may be dissolved or dispersed within the lipid core, the amphipathic lipid monolayer, or even the surface of the microemulsion. Compositions according to the present invention also comprise at least one lipidized polymer, preferably a lipidized protein or polypeptide, wherein at least a portion of said lipidized polymer is dissolved or dispersed within the amphipathic lipid monolayer and/or core lipid and at least a portion of the polymer is associated with the surface of the microemulsion.

Compositions according to the present invention may optionally comprise additional components such as fatty acids, steroid compounds and other compounds which may be added to the amphipathic lipid monolayer to modify the chemical and/or physical characteristics of the microemulsion.

Carriers according to the present invention may be used to mimic naturally occurring or native lipoprotein. The present approach may be used to accommodate large numbers of bioactive agents of varying physicochemical characteristics which are difficult to administer with acceptable bioavailability and pharmacokinetics. The present carriers may be used to dramatically increase the bioavailability of a desired agent and in certain embodiments, to enhance delivery of agents to a specific site of activity within the patient.

The present compositions therefore comprise a microemulsion as a carrier for a bioactive agent, said microemuslion comprising a lipid core which is surrounded by a monolayer of at least one amphipathic lipid and at least one lipidized polymer, the lipidized polymer preferably comprising a lipidized protein or polypeptide in combination with a bioactive agent which is dissolved within or dispersed into one or more of the lipid core or the amphipathic lipid or which is associated with the surface of the microemulsion. The bioactive agent may be any agent which exhibits a biological or pharmacological effect in a biological system, and preferably is a drug to be delivered to treat a condition or disease in a patient. More preferred bioactive agents include hydrophobic neutral or amphipathic drugs, especially those which may be more difficult to deliver because of their hydrophobic properties which severely limits water solubility, even more preferably anti-cancer or neoplastic agents.

Methods for enhancing the bioavailability of bioactive agents generally, and in particular, to specific sites within the patient using the compositions according to the present invention, represents an additional aspect of the present invention.

Detailed Description of the Invention

The following terms shall be used throughout the specification to describe the present invention.

The term "microemulsion" as used herein shall refer to an oil in water emulsion system with a small size (generally, having a median particle diameter in the sub-micron range, preferably ranging in size from about 1 to about 950 nanometers in diameter, more preferably about 5 to about 750 nanometers in diameter, even more preferably about 10 to about 500 nanometers in diameter, even more preferably about 25 to about 150 nanometers in diameter) which resembles the emulsion droplet in a natural or native lipoprotein, preferably a low density lipoprotein. The size of the microemulsion will generally depend upon the amphipathic lipid used to form the monolayer which surrounds the lipid core as well as the relative concentration of lipid in the lipid core to amphipathic lipid which surrounds the lipid core. The microemulsion according to the present invention is generally a spherical particle and is similar in size and shape to the lipid portion of a natural lipoprotein. The term "carrier" shall refer to the microemulsion which preferably includes a lipidized polymer, but excludes the bioactive agent. Note that the use of the terms "microemulsion", "submicron emulsion", "phospholipid submicron emulsion", "lipoprotein-resembling submicron emulsion" and lipoprotein-resembling particles" are synonymous terms used throughout the specification to describe emulsion system according to the present invention.

The term "bioactive agent" is used throughout the specification to describe a chemical compound which produces a biological effect in a biological system, preferably a mammalian system. Bioactive agents include drugs, toxins, pesticides and any number of compounds which produce a pharmacological response in a patient. Preferably, bioactive agents according to the present invention include any one or more compounds which are drugs and which may be used to treat a condition or disease state in a patient, in particular, a human patient. Bioactive agents include a broad range of compounds including, for example, anesthetics, systemic antibiotics, antiparasitics, systemic quinolones, anti-infectives, anti-

inflammatories, aminoglycosides, cephalosporins, penicillins, antidotes, anti-cholinesterases, metal poisoning antidotes, antineoplastics, 5'-fluorouracil, cytotoxic agents, hormones, steroids, immunomodulators, cytokines, systemic antivirals, systemic antifungals, biologicals, alpha-antitrypsin, bone metabolism regulators, hypercalcemic agent, cardiovascular agents, beta blockers, cerebral vasodilators, cerebral metabolic enhancers, cholinesterase inhibitors, colony stimulating factors, granulocyte-colony stimulating factors, granulocyte macrophage-colony stimulating factors, vasopressors, local diabetic agents, diagnostics such as CT scan enhancers and angiocardiography agents, adenosine deaminase deficiency agents, gonadotropin inhibitors, adrenal cortical steroid inhibitors, gonadotropin releasing hormone stimulant, urofollitropins, muscle relaxants such as neuromuscular blocking agents, prostaglandin analogs, prostaglandins, prostaglandin inhibitors, respiratory therapy agents, anticholinergics, beta andrenergic stimulators, sympathomimetics, and thrombolytics, antithrobotics, anticoagulants, antibiotics antiplatelet agents, thrombolytics, antiproliferatives, steroidal and nonsteroidal antiinflammatories, agents that inhibit hyperplasia and in particular restenosis, smooth muscle cell inhibitors, growth factors, growth factor inhibitors, cell adhesion inhibitors, cell adhesion promoters and drugs that may enhance the formation of healthy neointimal tissue, including endothelial cell regeneration agents, clonidine, estradiol, nicotine, nitroglycerin, and scopolamine, all of which are commercially available in the form of transdermal delivery devices. Others include antiinflammatory drugs, both steroidal (e.g., hydrocortisone, prednisolone, triamcinolone) and nonsteroidal (e.g., naproxen, piroxicam); antibacterials (e.g., penicillins such as penicillin V, cephalosporins such as cephalexin, erythromycin, tetracycline, gentamycin, sulfathiazole, nitrofurantoin, and quinolones such as norfloxacin, flumequine, and ibafloxacin); antiprotazoals (e.g., metronidazole); antifungals (e.g., nystatin); coronary vasodilators (e.g., nitroglycerin); calcium channel blockers (e.g., nifedipine, diltiazem); bronchodilators (e.g., theophylline, pirbuterol, salmeterol, isoproterenol); enzyme inhibitors such as collagenase inhibitors, protease inhibitors, elastase inhibitors, lipoxygenase inhibitors and angiotensin converting enzyme inhibitors (e.g., captopril, lisinopril); other antihypertensives (e.g., propranolol); leukotriene antagonists (e.g., ICI 204,219); anti-ulceratives such as H2 antagonists; steroidal hormones (e.g., progesterone, testosterone, estradiol, levonorgestrel); antivirals and/or immunomodulators (e.g., 1-isobutyl-1H-imidazo[4,5-c]quinolin-4-amine, 1-(2-hydroxy-2-methylpropyl)-1H-imidazo[4,5-c]quinoline-4-amine, and other compounds disclosed in U.S. Pat. No. 4,689,338, incorporated herein by reference, acyclovir); local anesthetics (e.g., benzocaine, propofol); cardiotonics (e.g., digitalis, digoxin); antitussives (e.g., codeine, dextromethorphan); antihistamines (e.g.,

diphenhydramine, chlorpheniramine, terfenadine); narcotic analgesics (e.g., morphine, fentanyl); peptide hormones (e.g., human or animal growth hormones, LHRH); cardioactive products such as atriopeptides; proteinaceous products (e.g., insulin); enzymes (e.g., anti-plaque enzymes, lysozyme, dextranase); antinauseants (e.g., scopolomine); anticonvulsants (e.g., carbamazine); immunosuppressives (e.g., cyclosporine); psychotherapeutics (e.g., diazepam); sedatives (e.g., phenobarbital); hypnotics; anticoagulants (e.g., heparin); analgesics (e.g., acetaminophen); antimigraine agents (e.g., ergotamine, melatonin, sumatriptan); antiarrhythmic agents (e.g., flecainide); antiemetics (e.g., metaclopromide, ondansetron); anticancer agents (e.g., methotrexate); neurologic agents such as anxiolytic drugs; hemostatics; anti-obesity agents; antigout agents; antianxiety agents; antiinflammatory agents; hormones; immunosuppressive agents; hyplipedmic agents; antiparkinson agents; antifungal agents; analgesics; antimanic agents; antipyretics; antiarthritic agents; antiplatetet agents; anticonvulsants; antidiabetic agents, anticoagulants, antiarrhythmics, antianginal agents; and the like, as well as pharmaceutically acceptable salts, esters, solvates and clathrates thereof., among numerous others.

Bioactive agents preferably comprise about 0.05% to about 50% by weight of the final composition (which weight includes the carrier, lipidized polymer and drugs in the final composition) according to the present invention, more preferably about 1% to about 35% by weight of the final composition. In certain preferred aspects according to the present invention, an amount of drug ranging from about 1% to about 15% by weight of the final composition is preferred. The amount of bioactive agent incorporated into the final composition will reflect the componentry of the microemulsion, the lipidized polymer used (which amount may vary greatly in the present compositions), the effective amount of agent required to elicit its intended effect in the patient. One of ordinary skill will be able to readily vary the componentry of the microemulsion, the amount and type of lipidized polymer in conjunction with the physicochemical properties of the bioactive agent in providing compositions according to the present invention.

The term "patient" is used throughout the specification to describe an animal, preferably a mammal and especially a human, to whom treatment, including prophylactic treatment, with the pharmaceutical compositions according to the present invention is provided.

The term "effective amount" is used throughout the specification to describe concentrations or amounts of composition according to the present invention which may be used to produce a favorable change in a disease or condition treated or to otherwise effect a biological, including a pharmacological, result. With respect to individual components which may comprise the final compositions according to the present invention, the term effective amount is used to describe that amount of a component which are included in compositions according to the present invention in order to produce an intended effect.

The term "amphipathic lipid" as used herein shall mean any suitable material of lipid or of a hydrophobic nature, preferably a phospholipid which can be used to create a monolayer which surrounds the lipid core in microemulsions according to the present invention. Generally, an amphipathic lipid has a hydrophobic neutral portion at one end of the molecule and a hydrophilic, often charged (often, anionic) portion at the other end of the molecule. In certain preferred aspects according to the present invention which relate to the delivery of DNA, the inclusion of positively charged lipids, with or without lipidized protein or polymer, may aid in the delivery of DNA using the microemulsion compositions according to the present invention. In the present invention, the amphipathic lipid produces a monolayer such that a hydrophobic portion of the amphipathic lipid material orients toward the interior of the microemulsion or lipid core, while a hydrophilic portion orients toward the aqueous phase or surface of the microemulsion. Lipids at the surface of the microemulsion may be charged (positive or negative) or neutral. Although any number of amphipathic lipids can be used in the present invention, including for example, the sodium and potassium salts of fatty acids, preferred amphipathic lipids include the phospholipids such as phosphatidylcholine (PC), cephalin, isolecithin (lysophosphatidyl choline), phosphatidylethanolamine (PE), distearoylphosphatidylcholine (DSPC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), sphingomyelin (SPM), and the like, alone or in combination. The phospholipids can be synthetic or derived from natural sources such as egg or soy. Some synthetic phospholipids which can be used in the present invention include, for example, dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG). The phospholipids which may be used in the present invention include chemical modified, conjugated or polymerized phospholipids such as pegylated phospholipids (which contains polyethylene glycol chains).

The amount of phospholipid which can be used in compositions according to the present invention is that amount effective to form a microemulsion in combination with lipid and, in most instances, bioactive agent. Preferably, the amount of phospholipid ranges from about 3% to about 75% by weight, more preferably about 15% to about 65% by weight of the microemulsion (i.e., excluding the lipidized polymer component) composition which weight includes lipid, phospholipid, optional steroidal component and bioactive agent, but which weight excludes lipidized polymer and any aqueous phase associated with the microemulsion in the final delivered composition. Preferred phospholipids include a mixture of egg phosphatidyl choline (22.7%) and lyosphophosphatidyl choline (2.3%) for a total phospholipid content of about 25% by weight. In addition to phospholipid in the phospholipid monolayer, a steroidal component may also be included in an amount ranging from about 0% to about 25% by weight of the phospholipid content of the monolayer, preferably, at least about 0.05% within this range. The amount of lipidized polymer which may be included in final compositions according to the present invention ranges from about 0.01% to about 65% by weight or more, preferably about 0.5% to about 50% by weight, even more preferably about 1% to about 35% by weight and even more preferably about 2.5% to about 15-20% by weight, depending on the polymer used. In a number of instances, where, for example, lysozyme is used, the amount of protein associated with the microemulsion will range from about 3-10% or more by weight, preferably about 5.0-8.0% or more by weight of the final composition. In certain preferred embodiments related to microemulsion compositions according to the present invention which aid in the delivery of DNA, the inclusion or use of positively charged lipids is preferred.

The amphipathic lipid monolayer (as well as the lipid core) may also include steroidal components such as cholesterol, polyethylene glycol derivatives of cholesterol (PEG-cholesterols), coprostanol, cholestanol, or cholestane, or combinations of these steroids. Preferred steroids include cholesterol and cholesterol-like steroids such as cholestane. These steroids may be neutral or charged, especially where they are found at the surface of the microemulsion. It is noted here that certain positively charged steroids included in the formulations with or without lipidized protein or polymer may aid in the delivery of DNA using the microemulsion compositions according to the present invention. In addition, steroid esters (preferably, C_1 to C_{24} steroid esters, which may be formed by reacting a free hydroxyl group on the steroid with an organic acid, preferably a C_{12} to C_{24} fatty acid, or alternatively, by reacting a carboxylic acid group on the steroid with an alcohol, preferably, a

C_{12} to C_{24} fatty alcohol) such as, for example, cholesterol oleate, among numerous others, may also be included. The steroid compounds may be added in amounts ranging from about 0.005% to about 20% by weight of the amphipathic lipid used to form the monolayer of the microemulsion. Steroid components may be added to the microemulsion primarily to stabilize the microemulsion as well as to change other characteristics of the compositions. The amphipathic monolayer of the present invention may also contain glycolipids.

The term "lipids" or "lipid core" is used throughout the specification to describe a core of the microemulsion which is made from a pharmaceutically acceptable lipid material, which is generally defined as a lubricious and hydrophobic material which is obtained from animal, vegetable or mineral matter or is synthetically prepared. Oils for use in the present invention are generally neutral (i.e., uncharged) and are generally neither acidic nor basic. Oils for use in the present invention may include petroleum-based oil derivatives such as purified petrolatum and mineral oil (especially when the microemulsions are formulated for use in cosmetic and/or topical applications), but in general, the lipids used in the present invention are preferably mono-, di- and triglycerides or other neutral, esterified fatty acid compounds.

Petroleum-derived lipids or oils for use in the present invention include aliphatic or wax-based oils and mixed base oils and may include relatively polar and non-polar oils. "Non-polar" oils are generally oils such as petrolatum or mineral oil or its derivatives which are hydrocarbons and are more hydrophobic and lipophilic compared to certain natural and synthetic oils, such as mono-, di- or triglycerides or other esters, which may be referred to as "polar" oils. It is understood that within the class of lipids, that the use of the terms "non-polar" and "polar" are relative within this very hydrophobic and lipophilic class, and all of the oils tend to be much more hydrophobic and lipophilic than the hydrophilic portion of the amphipathic lipids which are also used in the present invention.

In addition to the above-described oils, certain pharmaceutically acceptable essential oils derived from plants such as volatile liquids derived from flowers, stems and leaves and other parts of the plant which may include terpenoids and other natural products including triglycerides may also be considered lipids for purposes of the present invention. The use of naturally occurring triglycerides as lipids for use in the present invention is clearly preferred.

Preferred lipids or oils for use in the present invention may include, for example, mono-, di and triglycerides which may be natural or synthetic (derived from esterification of glycerol and at least one or more saturated or unsaturated organic acid, such as, for example, butyric, caproic, palmitic, stearic, oleic, linoleic or linolenic acids, among numerous others, preferably a fatty organic acid, comprising between 8 and 26 carbon atoms). Glyceride esters for use in the present invention include vegetable oils derived chiefly from seeds, nuts and vegetables and include drying oils, for example, linseed, iticica and tung, among others; semi-drying oils, for example, soybean, sunflower, safflower, palm, poppy seed , corn, sesame seed, olive, canola and cottonseed oil; non-drying oils, for example castor and coconut oil, among numerous others. Hydrogenated vegetable oils also may be used in the present invention. Animal oils are also contemplated for use as glyceride esters and include, for example, fats such as tallow, lard and stearin and liquid fats, such as fish oils, fish liver oils and other animal oils, including sperm oil, and vitamins such as the tocopherols, among numerous others. In addition, a number of other oils may be used, including C_{12} to C_{30} (or higher) fatty esters (other than the glyceride esters, which are described above). Triolein is a preferred lipid for use in the present invention.

The ratio of the amount of lipid to drug in the present compositions ranges from about 50:1 to about 1:10, preferably about 20:1 to about 1:2, depending upon the hydrophobicity (lipophilicity) of the bioactive agent to be used. The amount of lipid and drug generally ranges from about 5% to about 85% by weight of the microemulsion (i.e., the composition without the lipidized polymer), preferably about 10% to about 75% by weight. The preferred ratio is approximately 70% by weight of the microemulsion, with triolein as the preferred lipid used.

The lipid core of the microemulsion may contain a steroidal component (preferably, an esterified steroidal component such as cholesterol oleate or a similar ester of cholesterol) in the range of about 0 to about 50% by weight of the core (lipid plus hydrophobic bioactive agent).

The term "lipidized polymer" is used to describe a primarily hydrophilic polymer of varying chemical composition which has been modified (preferably through covalent bonding) to contain fatty acid, phospholipid or other hydrophobic (such as a hydrophobic steroidal component or other hydrophobic moiety) residues in order such that the fatty acid or

hydrophobic portion of the lipidized polymer will readily interact with (i.e., dissolve or disperse into) the lipid component of the microemulsion (whether that lipid component is in the lipid core or the lipid portion of the amphipathic lipid monolayer) while the non-lipid component of the polymer will associate with the surface of the microemulsion.

Lipidized polymers according to the present invention are readily formed by reacting fatty acids or phospholipids (or their corresponding activated esters or equivalent groups such as acyl halides) of varying lengths with one or more nucleophilic positions on the polymer, such as a free amine, hydroxyl or thiol containing group, preferably an amine group, which results in formation of an amide. Preferably, the polymer from which the lipidized polymer is prepared is a protein or polypeptide such as polylysine or other polypeptide containing at least 3 amino acid units, and more preferably is a protein or polypeptide which functions to bind to a target at a cell site within the patient or alternatively, functions to bind other components such as other proteins, polypeptides and bioactive agents such as drugs, among others, to be delivered by the microemulsion.

Alternatively, lipidized polymer (preferably, protein) may also be carried out at the surface of the preformed microemulsion system by using reactive phospholipid (an activated form of phospholipid wherein the phosphate group is activated, for example, as an activated phosphate ester), cholesterol, fatty acids, or other hydrophobic surfactants while formulating the microemulsion, followed by incubation of the microemulsion with the protein for the reaction to occur.

In the case of the use of protein, in general, the attachment of a fatty or other hydrophobic chain to the protein is very much needed in order to ensure the association between the microemulsion components and the protein.

The preferred chemical lipidization of the protein according to the present invention maintains the biological integrity or function of the protein. Other procedures for lipidization may also be used, including, for example, a reverse micelle system for protein hydrophobization as described by Kabanov et.al (*Biol. Mem. Volume 2(10), 1989, pp.1769-1785*), conjugation under organic solvent conditions as described by Hashimoto, et.al (*Pharm.Res, Vol.6, No.2, 1989*), conjugation under aqueous solutions using micellar suspension as described by Huang, et. al (*Biol.Chem. Vol 255,No.17, pp 8015-8018, 1980*).

The method used to prepare compositions according to the present invention depends upon the utilization of a mixture of aqueous and organic solvent (dimethyl formamide) forming a one-phase reaction media to ensure the solubilization of the protein and the fatty acid reagent. One or more of other organic solvents may also be used. The degree of lipidization of the polymer/protein component may also be controlled by the ratio of fatty acid reagent / protein (or other polymer). Numerous proteins and polymers can be lipidized in a similar manner.

Preferably, the polymer is a biological polymer, such as a protein or polypeptide, but also may comprise a polynucleotide (preferably, deoxyribonucleic acid or DNA, because these polymers tend to be less labile than are polymers of ribonucleic acid) of at least three nucleotide units. In general, polymers which are used in the present invention which are other than a protein , polypeptide or polynucleotide are used in cosmetic or topical applications or applications other than for the systemic delivery of a drug. Of course, biodegradable and/or bioresorbable synthetic hydrophilic polymers such as polyesters including polylactic acid and polyglycolic acid polymers, among others, including polyvinylpyrrolidone, may also be used in the present invention in formulations to be used for systemic delivery of drugs. The term "protein" shall mean any protein, regardless of whether that protein is a polypeptide, a glycoprotein or a related protein.

Preferred proteins or polypeptides which may be used in the present invention include, for example, enzymes, cell surface proteins, hormones, antibodies, growth factors, clotting factors, neuroproteins, tumor suppressors, toxins, antigens and epitopes of antigens, apolipoproteins including for example, apolipoprotein B100, apolipotroeint E, endogenous or exogenous tumor antigenic proteins, among numerous others, including bioinvasive molecules (like bacterial invasives), lectins, lectin-like molecules, bacterial toxins (cholera toxin) and macromolecules with bioadhesive properties. Proteins can be chosen to have bio-targeting ability (hereinafter, referred to as "targeting proteins") in order to direct the microemulsion particle to different biological, especially tissue sites. This approach may dramatically increase the delivery of a bioactive agent to a particular site within a patient in order to enhance therapy, especially where the bioactive agent is seen to have tissue-specific or tissue-selective activity. Preferred targeting proteins or polypeptides include, for example, immunoglobulins or appropriate epitopes, transferrin, avidin, hormones, enzymes, integrin,

polylysine, Apolipoprotein E, monoclonal antibodies, and the like. Also appropriate for use in the present invention is any part of the proteins or a synthetic analogue. The preferred reaction conditions are described in detail hereinbelow. Still other preferred proteins include receptors such as hormone receptors including insulin receptors and growth factor receptors, among others. Especially preferred proteins include, for example lysozyme and avidin. In certain preferred embodiments, the inclusion of apolipoprotein B100, apolipoprotein E or apolipoprotein, in combination with one or more targeting proteins such as avidin or lysozyme, may provide a targeting approach to the delivery of drug through the LDL pathway or to other tissues within the body.

In a particularly preferred embodiment according to the present invention, avidin is lipidized using a C_8-C_{52} fatty acid, preferably a fatty acid containing between eight and 26 carbon atoms, including such fatty acids as palmitic, stearic, oleic, linoleic or linolenic acids, among numerous others. Avidin, a glycoprotein, is preferred in certain instances, because once it is lipidized and formulated into the present microemulsion compositions, it is capable of binding to any biotinylated component and allows a biotinylated component to be attached to the microemulsion through a biotin-avidin complex. The lipidized avidin embodiment of the present invention is especially useful for attaching biotinylated antibodies, as well as a number of biotinylated proteins (such as enzymes, toxins and antigens, among others) as well as biotinylated oligo- and polynucleotides to the microemulsion according to the present invention.

This embodiment is advantageous because it results in a product which can function as a universal station for the attachment of various biotinylated proteins (enzymes, toxins, antibodies, etc.), polymers or drugs. The biotin-avidin embodiment according to the present invention is particularly advantageous approach when attaching an antibody to the microemulsion because it results in standardization, avoids the potential problems of non-selective modification of the antibody and disorientation of the antibody in the microemulsion arising from direct modification on the antibody (potentially resulting in the antibody binding site being buried inside the microemulsion and being unavailable for binding). See atttached figure 4.

The synthetic lipoprotein according to the present invention is distinguishable from the natural lipoprotein in that the natural lipoprotein has an apolipoprotein (for example,

apoplipoprotein B100 in the case of low density lipoprotein) associated with it, while the lipoprotein according to the present invention has a protein which has been chemically modified to attach hydrophobic groups such as fatty acid chains in order to more closely have the protein associate with the lipid portion of the synthetic lipoprotein. This allows the protein to lower the surface tension at the microemulsion surface and thereof, enhancing the stability of the microemulsion. In the case of the natural low density lipoprotein, it is believed that the association of apolipoprotein B100 with the lipid portion serves as a component for receptor interaction. The protein penetrates the whole natural microemulsion system to enhance the LDL particle stability. This is due to its chemical nature of having a hydrophobic region that spans the core of the LDL microemulsion and a hydrophilic region which protrudes to the surface of the LDL microemulsion which also serves as a searching tool for LDL receptors.

In certain preferred aspects according to the present invention which include a lipidized polymer in effective amounts, the resulting microemulsion exhibits favorable characteristics of enhanced stability, in comparison to similar microemulsions which exclude lipidized polymer, preferably lipidized protein. Enhanced stability within this context shall mean that the resulting microemulsion particles exhibit a consistent size (generally, approximately within the original size estimate without appreciable or substantial change), degradation is substantially reduced and aggregation is substantially reduced or preferably eliminated. These favorable characteristics result in the microemulsion compositions according to the present invention being storage stable in comparison to prior art compositions even in final emulsion form (i.e., when formulated in combination with an aqueous solution prior to administration to a patient).

Another aspect of the present invention relates to a method or methods for transferring an active agent within the microemulsion of the present invention to LDL or other natural lipoprotein (the term "natural lipoprotein" as used herein describing lipoproteins which are found naturally in the patient as opposed to the synthetic lipoproteins which may be used in the present invention), even in the presence of serum proteins. The characteristic of the present invention in this regard is a particularly beneficial and efficient method for transferring bioactive agents of the present invention into LDL or other natural lipoproteins including, for example HDL (high density lipoprotein), VLDL (very low density lipoproteins), IDL (intermediate density lipoproteins) and chylomicrons and results in the

agent being delivered to sites within the patient more efficiently than using prior art methods, including liposomes. This method may be used *in vitro* (generally, in the presence of an effective amount of a lipid transfer protein) to produce natural LDL's or other lipoproteins containing bioactive agents which may subsequently be used to administer bioactive agents to a patient or alternatively, the method may be used *in vivo*, to enhance the delivery of bioactive agents into cellular active sites by transferring the bioactive agent to natural LDL or other lipoproteins which may be more easily "taken up" into the cells of the patient, where the agent may produce a maximal affect. While not being limited by way of theory or mechanism, it is believed that transfer occurs from the microemulsions according to the present invention to the LDL or other natural lipoprotein via lipid transfer proteins such as cholesteryl ester transfer protein (CETP), phospholipid exchange protein and lipase. The term "lipid transfer protein" is used to describe a protein which facilitates the transfer of a lipid from the microemulsion according to the present invention to a natural lipoprotein or LDL.

Preferred embodiments of the lipoprotein according to the present invention utilize proteins with additional biological targeting capability which makes the system capable of targeted delivery of pharmaceutical agents to different biological sites in the body. For example, the use of avidin to attach biotinylated antibodies and other components as well as the use of lysozyme and hormones which may be used to attach to receptors at desired locations in the body, represents an additional approach to targeting of the present synthetic lipoproteins for delivery of drugs to patients.

The term "extended delivery" as used herein is understood to mean the release of therapeutic agents from microemulsions encapsulation over a period in excess of what would normally occur without the presence of stable microemulsions and generally in about 24 hours and in some embodiments as long as about 2 to 3 weeks.

"Structural integrity of microemulsions" as used herein shall mean the substantial maintenance of the pharmaceutical activity of the encapsulated substance during a period of extended delivery. This structural integrity is presumed to arise from the incorporation of lipidized polymer into the microemulsion formulations according to the present invention and the substantial maintenance of an entrapped bioactive agent, preferably a drug, for the period of extended delivery. Structural integrity may be imparted by adding lipidized polymer to the

microemulsion in order to maintain the required microemulsion structure when challenged by the physiological conditions present in the subject animal. Alternatively, in some instances the inclusion of higher weight percentages of a steroidal component may also add to the structural integrity.

Compositions according to the present invention may be used to facilitate targeted therapy, especially targeted gene therapy. Lipidized polymers such as poly-L-lysine or polyethylenimine, among other cationic polymers, especially including polyamines, can be preparated in lipidized form and formulated such that the protein is associated with the surface of the microemulsion. These cationic polymers can be advantageously used for gene delivery since cationic polymers are known to complex with DNA through electrical attraction (interaction) between the negatively charged DNA and the positively charged polymers. (See attached Figure 3).

While not being limited by way of theory, it is believed that the artificial LDL system of the present invention may also behave as native LDL by acquiring apolipoprotein E from the plasma. Embodiments of the present invention which incorporate apoliporoteins such as apolipoprotein B100 or apopliporotein E, in combination with lipidized protein represent further embodiments of the present invention.

Particular benefits of the compositions according to the present invention include the substantial enhancement of drug delivery to sites within the patient's body (targeting), the enhancement of drug entrapment upon formation of the microemulsions, the enhancement of the amount of drug per amount of lipid (loading), and the amount of hydrophobic drug which can be accommodated by this mode of delivery. Additional benefits of the present invention include the extended elaboration that microemulsion dosage forms according to the present invention exhibit when administered parenterally, especially via intramuscular, intramammary, intradermal, intraperitoneal, intra-ocular or subcutaneous routes. Topical dosage forms may also be formulated to exhibit extended release characteristics.

In a microemulsion-drug delivery system according to the present invention,, the therapeutic agent preferably is encapsulated in the microemulsion (preferably, in the lipid core or amphipathic lipid monolayer or alternatively, at the surface of the microemulsion), associated with lipidized polymer and then administered to the subject being treated.

Preferably, in certain embodiments, in order to take advantage of the extended elaboration characteristics of the dosage forms of the present invention, the dosage forms are administered parenterally via an intramuscular or subcutaneous route. The dosage forms of the present invention may also be administered via an intra-ocular, intramammary, intradermal or intraperitoneal route to produce a therapeutic dosage form having the characteristics of extended elaboration. In addition to the parenteral dosage forms, certain dosage forms of the present invention may be utilized in topical or oral formulations.

Compositions according to the present invention may be formed in the presence of solvent or an aqueous medium. As for the technique of preparation of the microemulsion, it is preferred to use a probe sonicator, such as the Branson 450 to form the microemulsion. Other homogenizing devices may also be used such as high shear mixers, high-pressure homogenizers, high shear impellers and the like. The compositions may be used immediately or stored prior to use.

Alternatively, the composition solutions may be dehydrated, thereby enabling storage for extended periods of time until use. Standard freeze-drying equipment or equivalent apparatus may be used to dehydrate the solutions containing the microemulsions. The solutions may also be dehydrated simply by placing them under reduced pressure. Alternatively, the microemulsions and their surrounding medium can be frozen in liquid nitrogen prior to dehydration. Dehydration with prior freezing may be performed in the presence of one or more protective sugars in the preparation including, for example, trehalose, maltose, sucrose, glucose, lactose and dextran, among others. When the dehydrated microemulsions solution is to be used, rehydration is accomplished by methods which include simply adding an aqueous solution, e.g., distilled water, to the microemulsions and allowing them to reformulate in solution.

In an alternative method embodiment, microemulsions comprising a bioactive agent according to the present invention are formulated and then incubated in the presence of a natural lipoprotein, preferably an LDL, and a lipid transfer protein, preferably cholesteryl ester transfer protein (CETP), phospholipid exchange protein (PEP) or lipase at a concentration and temperature (generally, within a range of approximately 30-40°C, preferably about 37°C) under conditions which allow enzymatic reactions to take place in

order to promote transfer of the bioactive agent in the microemulsion to the natural lipoprotein. In this method aspect according to the present invention, the resulting natural lipoprotein or LDL containing bioactive agent may be separated from the microemulsion and administered to the patient, or administered to the patient in combination with the microemulsion. A method which results in at least about 5% by weight of the bioactive agent in the microemulsion being transferred to the natural lipoprotein is preferred, at least 10% by weight of the bioactive agent in the microemulsion being transferred is more preferable and at least about 15-18% by weight of the bioactive agent being transferred is even more preferable.

The compositions according to the present invention are generally administered in association or in admixture with a pharmaceutically acceptable carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

Bioactive agents for incorporation into microemulsions include, for example, those which have been described in the present specification as well as additional agents, not otherwise specified. The present compositions may accommodate a broad range of chemical characteristics ranging from hydrophobic (lipophilic) to hydrophilic (lipophobic) and are particularly appropriate for agents which are lipid soluble, but are difficult to deliver because of their hydrophobicity. Particular bioactive agents are understood to include analogues and derivatives of such agents including biologically active fragments unless otherwise indicated.

Pharmaceutical dosage forms of the present inventions may be comprised of microemulsions comprising a bioactive agent (preferably also including a lipidized protein associated therewith) and optionally, any suitable pharmaceutical carrier. A preferred class of carrier is aqueous including both distilled water and isotonic saline. Administration of high integrity microemulsions (i.e., those microemulsions associated with a lipidized protein) according to the present invention may be accomplished by any usual route with particular reference to the preferred routes of administration.

Preferred routes of parenteral administration as used herein include intracranially, intramuscular, intramammary, intraperitoneal, subcutaneous and intra-ocular administration. However, dosages adapted to parenteral administration may be used in a variety of administration methods, especially including topical and oral administration.

Having generally described the invention, reference is now made to the following specific examples which are intended to illustrate preferred and other embodiments and comparisons. The included examples are not to be construed as limiting the scope of this invention as is more broadly set forth above and in the appended claims.

Examples

Example 1 Lipoprotein-Resembling Phospholipid-submicron Emulsion for Cholesterol-Based Drug Targeting, BCH

The objective of this experiment was to develop and evaluate lipoprotein-resembling phospholipid-submicron emulsion (PSME) as a carrier system for new cholesterol-based compounds for targeted delivery to cancer cells. BCH, a boronated cholesterol compound for boron neutron capture therapy (BNCT), was originally developed in our laboratory to mimic the cholesterol esters present in the LDL and to follow a similar pathway of cholesterol transport into the rapidly dividing cancer cells. The lipoprotein-resembling system was designed to solubilize and facilitate BCH delivery to cancer cells. BCH-containing PSME was prepared by sonication. Stock solutions of individual lipid and BCH were prepared in chloroform. Various lipids and BCH were mixed and the mixture was composed of the following ratio (w/w): Triolein: egg phosphatidylcholine: lysophosphatidylcholine: cholesterol oleate: cholesterol: BCH, 70: 22.7: 2.3: 3.0: 2.0: 2.0, respectively. All components were combined and chloroform was evaporated under a stream of nitrogen. The preparation was then desiccated overnight at 4°C to remove residual solvent. Following addition of 10 ml of 2.4 M NaCl for 102 mg of lipid and BCH mixture, the preparation was sonicated under nitrogen for 30 min using a probe sonicator (Branson Sonifier 450) at output 5, while the temperature was maintained at 55°C. Inductively coupled plasma (ICP) and thin layer chromatography (TLC) were used to monitor possible degradation of BCH with the sonication condition.

The lipid structure of PSME and location of BCH in the formulation were assessed based on experimental results. Density gradient ultracentrifugation fractionated the emulsion into 3 particle size populations with structures and compositions resembling native lipoproteins. Chemical compositions and particle sizes of different PSME particles were determined. The following table shows the chemical composition:

Table 1: Composition of different fractions of BCH-containing PSME

PSME Fraction	Particle size	BCH (%w/w)	Cholesterol (%w/w)	Cholesteryl oleate (%w/w)	Phospholipids (%w/w)	Triolein (%w/w)
First	161±2	2.4±0.1	1.9±.1	3.0±0.2	6.5±0.6	86.3±0.5
Second	76±1	2.2±0.1	2.3±0.1	2.8±0.1	12.6±0.4	80.1±0.2
Third	40±3	1.8±0.1	3.1±0.2	2.5±0.1	27.4±0.4	65.3±0.9
Fourth	NA	0.5±0.1	3.7±0.2	0.3±0.1	89.3±0.8	6.1±0.1

[a] All value are means ± SEM of three experiments.

According to the chemical composition, the location of BCH, with respect to other lipids, was confirmed to be in the core of these lipoprotein particles the same as triolein and cholesteryl oleate, while phospholipids and cholesterol were present at the surface (See figures 1 and 2). This indicates the composition and structure similarity between these submicron particles and native lipoproteins.

The Stability of the BCH-containing PSME formulation was also studied. Dialyzed BCH-containing PSME fractions were incubated at room temperature and frozen at 4°C to study any possible hydrolysis of the drug (BCH) and the stability of these different particles. Samples were collected at day 1, 3, 10, and 38 for each fraction at different incubation temperatures and analyzed for BCH by HPLC. Particle size of each sample was also measured by photon correlation spectroscopy.

After 38 days the BCH and the formulation appeared to be unchanged. The particles size remained without any significant change during this period. HPLC analysis of the BCH showed that after 38 days at 4°C about 85% of the initial BCH was recovered from the formulation.

Cell culture data showed sufficient uptake of BCH in rat 9L glioma cells (it is an important requirement to have about 20 μg of ^{10}B per gram of cells to achieve successful BNCT) as shown in following table:

Table 2: BCH uptake in 9L glioma cell culture

Experiment	Initial concentration of B in the media (μg/ml)	Concentration of B in the cells after 18 hrs incubation (μg/g cells)
Control	0	0
Low concentration [a]	8.25	50±10
High concentration [a]	16.5	61±13

[a] All values are means ± SEM of three experiments.

The lipoprotein-resembling PSME appears to be a novel carrier system that can incorporate a cholesterol-based compound, interact with native LDL and sufficiently deliver the compound into cancer cells in vitro. The sonication method used in the preparation didn't affect the compound or the lipids during the formulation. The formulation appears to be stable with minimal degradation after 38days. Our study suggests that these PSME particles have similar lipid composition and structural organization as native lipoproteins with the ability to interact and associate with LDL *in vitro.*

Example 2: BCH distribution into human LDL and uptake by human glioma cells 767SF

The purpose of this experiment was to study the efficiency of delivering newly synthesized boronated cholesterol, BCH, for boron neutron capture therapy to human glioma cells 767 SF. The drug was incorporated in a lipoprotein-resembling submicron emulsion to benefit from the increased uptake of the cholesterol in these cells due to the increased demand of the building new cell membrane for these rapidly dividing cancer cells. Also the similarity in structure between these BCH-containing submicron emulsion and native lipoproteins may contribute the dynamic exchange and transfer of lipid between different

lipoproteins in the body and consequently the transfer of the boronated cholesterol to the cancer cells.

Methods: BCH-containing submicron emulsion was prepared by sonication at 55°C of various lipids, the natural component of native lipoproteins, along with the boronated cholesterol. In vitro transfer of BCH to LDL was evaluated in LPDS and PBS. BCH uptake by human glioma cells 767 SF was compared when FBS or LPDS were used in the culture media.

Results: After separating the submicron emulsion particles from the LDL, analysis of the LDL particles showed that about 18% of BCH originally incorporated in the second fraction of the submicron emulsion was transferred to the human LDL after 1hr incubation at 37°C in LPDS media. This transfer was not observed when the incubation was carried in PBS (no BCH was detected at the LDL fraction after incubation). This indicates that certain molecule in the serum, probably cholesteryl ester transfer protein CETP, was responsible for the interaction and transfer of BCH to the human LDL. This indicates also the unique resemblance between these submicron emulsion particles and serum lipoproteins as they exchange lipids in the presence of serum proteins. Lipid exchange between different classes of lipoproteins was established by others to be dependent on the presence of certain exchange proteins like CETP and phospholipid exchange protein. CETP was described as less selective to its substrate as it transfers not only cholesteryl esters (CE) but also triglycerides (Yokoyama et al, *Magnes.Res.*, 7, (2) 87-105 (1994). This can be advantageous since drug molecules of similar structure of CE may also be a substrate for this enzyme and be transferred to body's lipoproteins when incorporated in this submicron emulsion. Drug transfer to LDL can be another attribute of this formulation to selective delivery via the LDL pathway.

To further investigate the use of this formulation in vitro we studied the cellular uptake of BCH in the presence and absence of lipoproteins, in FBS and LPDS. Results showed that uptake in FBS (lipoproteins are present) was more than triple the amount when the media contained no lipoproteins (LPDS) as shown in the following table:

BCH uptake in 767SF glioma cell culture

Experiment	Initial concentration of B in the media (μg)	Concentration of B in the cells after 18 hrs incubation (μg/g cells)
Control	0	0
FBS [a]	38	57.6±15.5
LPDS [a]	38	14.7±3.5

[a] All values are means ± SEM of three experiments.

These results, in FBS, also meet the requirement for successful boron neutron therapy (>20μg B/gm cells).

Example 3: Amphotericin B incorporation in lipoprotein-resembling submicron emulsion:

Stock solutions of individual lipid were prepared in chloroform; Amphotericin B (AmpB) was dissolved in methanol. Various lipids and AmpB were mixed and the mixture was composed of the following in mg: Triolein: egg phosphatidylcholine: lysophosphatidylcholine: cholesterol oleate: cholesterol: AmpB, 70: 22.7: 2.3: 3.0: 2.0: 10, respectively. All components were combined and chloroform/methanol was evaporated under a stream of nitrogen. The preparation was then desiccated overnight at 4°C to remove residual solvent. Following addition of 10 ml of 2.4 M NaCl for 110 mg of lipid and AmpB mixture, the preparation was sonicated under nitrogen for 30 min using a probe sonicator (Branson Sonifier 450) at output 5, while the temperature was maintained at 55°C. Particles were separated and dialyzed as described before. AmpB was analyzed by spectrophotometry after suitable dilution in methanol at 404.5nm.

AmpB was successfully solubilized in the submicron emulsion system (2.33mg of the added AmpB was incorporated in the system). Most of the incorporated AmpB (72%) was recovered with the first fraction of the submicron emulsion, which had particle size of 177nm. AmpB is very effective antifungal drug; unfortunately it suffers from side effects when used parenterally (renal toxicity). Another limitation of this compound is its low water solubility. Incorporating AmpB in lipoprotein-resembling emulsion can lower its toxicity and solubilize the drug for successful parenteral administration.

Example 4- Lipidized Lysozyme

As for the second component of the artificial LDL system, the lipidized protein- we have conducted our experiment on lysozyme as a model protein for the chemical lipidization. The following study describes the chemical reaction for the lipidization and its detection, which can be carried out for virtually any other protein having nucleophilic amine, hydroxyl or thiol (preferably amine) groups as well. The objective was to chemically modify lysozyme by the addition of fatty chains of stearic acid to the lysine amino group present in the protein and the detect the chemical modification by MALDI-TOF.

Methods

Preparation of active ester

3.45 g N hydroxysuccinamide was dissolved in dry ethyl acetate (150ml). Then 8.53 g of stearic acid was added to that solution. A solution of dicyclohexylcarbodiimide (6.18 g) in dry ethyl acetate (10ml) was added to the above reaction mixture and left overnight at room temperature. Dicyclohexylurea was removed by filtration and the filtrate yielded white crystal of the stearic acid active ester under rotavapor. The crystals were then recrystalized in ethanol,

Lipidization of Lysozyme

10mg of the stearic acid active ester prepared above were dissolved in 1.5ml of dimethylformamide (DMF) and then dropwise added while shaking to 2.5ml solution containing 10mg lysozyme in distilled water. The mixture was left overnight at 37°C. The mixture was then dried under reduced pressure and redissolved in distilled water and passed through a 0.45 syringe filter. A control reaction was also conducted under the same conditions but without the addition of stearic acid ester to eliminate any effect of the reaction conditions on the molecular weight of lysozyme.

MALDI-TOF Analysis

The modified lysozyme sample was analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometry using a Bruker Reflex time

flight mass spectrometer (Billerica, MA) retrofitted with delayed extraction. The matrix was a saturated solution of 3.5 dimethoxy-4 hydroxycinamic acid (Aldrich, Milwaukee, WI) in a 50:50 mixture of water:acetonitrile with 0.1% triflouroacetic acid (TFA). The MALDI target was first spotted with nitrocellulose and allowed to dry. 2 (L of sample was applied next and dried. The sample was washed with cold water with 0.1% TFA. After washing two times, 0.5 (L of solvent was added to each sample. The spectrum was acquired in linear mode by averaging 26 laser shots and was externally calibrated using lysozyme MH+ and MH22+.

Results

The mass spectrum of the modified lysozyme showed an obvious increase in the molecular weight of lysozyme (figure 3). The shifting range was around 1000 da, which represents the attachment of three fatty chains of stearic acid. There was no effect of the reaction media on the molecular weight of lysozyme when no active ester was added.

Conclusions

Chemical lipidization of lysozyme was achieved. MALDI-TOF is an appropriate tool to evaluate the extent of reaction in producing lipidized protein. This method may be used readily to modify other proteins in a similar way.

Since the chemical lipidization is an important element to promote the attachment of proteins to the microemulsion system according to the present invention, one can readily lipidize virtually any protein in a similar manner. Other polymers may be modified in similar manner and used to target different tissues in the body.

Example 5: Lipidized polylysine and its association with lipoprotein-resembling submicron emulsion for gene delivery

This experiment describes the lipidization of positively charged polymer, polylysine, and its association with the lipoprotein-resembling submicron emulsion particles. Polylysine has been used to condense DNA through charge interaction. Cationic lipids in liposome formulation have been also used to carry DNA using the same concept of charge interaction. Unfortunately, cationic lipids are very toxic and their use is limited for in vitro transfection of

DNA. In this patent we describe a system, which utilize the positive charge of cationic polymers and the biocompatible formulation of lipoprotein resembling submicron emulsion system to deliver negatively charged DNA.

Methods:

Preparation of lipoprotein-resembling phospholipid submicron emulsion

Stock solutions of individual lipid were prepared in chloroform. Various lipids were mixed and the mixture was composed of the following ratio (w/w): Triolein: egg phosphatidylcholine: lysophosphatidylcholine: cholesterol oleate: cholesterol, 70: 22.7: 2.3: 3.0: 2.0, respectively (reference maranhoa and van Berkel). All components were combined and chloroform was evaporated under a stream of nitrogen. The preparation was then desiccated overnight at 4°C to remove residual solvent. Following addition of 10 ml of 2.4 M NaCl for 102 mg of lipid and BCH mixture, the preparation was sonicated under nitrogen for 30 min using a probe sonicator (Branson Sonifier 450) at output 5, while the temperature was maintained at 55°C.

Lipidization of poly-L-lysine

N-alkylation of poly lysine (PLL) was achieved as described by Kim et al., *J. Controlled Release,* 47, pp. 51-59 (1997), with modification. In brief, 30mg PLL was dissolved in 2ml DMSO. 10µl of triethylamine was added to the mixture. Palmitoyl chloride (20mg) was used to react with the ε-amino of the lysine in the poly lysine polymer. The mixture was allowed to react at room temperature for 2hrs. The mixture was filtered and acetone was added to the filtrate to precipitate the lipidized polymer, palmitoyl poly lysine. The product was dissolved in methanol, reprecipitated by acetone, and dried under vacuum overnight. The modified polymer was characterized by proton NMR.

Interaction of lipidized poly lysine with lipoprotein-resembling submicron emulsion

The fractions of different sizes of the phospholipid submicron emulsion were incubated individually (TEFF 50µl of each fraction or 20µl LDL+30µl PBS) with 100µg or 50µg of poly lysine or palmitoyl poly lysine for 1 hr in 2 ml PBS at 37°C with gentle shaking. Agarose gel electrophoresis was performed according to the method described by

Greenspan *et al., Electrophoresis*, 14, pp. 65-68 (1993) using Nile Red as the fluorescent dye to determine the electrophoretic mobility of the lipoprotein-resembling submicron emulsion and to examine its interactions with lipidized and native poly lysine. In brief, 0.6% agarose gel was prepared in 50 mM barbital buffer, pH 8.6. Five µl of Nile Red in acetone (100 µg/ml) was dried out in test tube. The incubation sample was then added to the tube individually (50 µl sample to each tube) and mixed until Nile Red was in solution. Five µl of sucrose solution (30%, w/v) was added. Each electrophoretic well was loaded with 11 µl of sample preparation. Electrophoresis was conducted for 1 hr at 56 V at room temperature. Different electrophoretic bands on the gel were visualized under UV lamp.

Degree of lipidized poly lysine association with PSME

In order to measure the amount of lipidized PLL associated with the submicron emulsion particles, (250µl of 2nd fraction blank containing 2.391 mg T/ml) of the second fraction were incubated with 2mg of lipidized poly lysine in 2ml PBS for 1 hr at 37°C with gentle shaking. The density of the mixture was adjusted to 1.08g/ml with solid KBr and placed in the bottom of 13.5ml centrifuge tubes; the remaining of the tube was filled with KBr solution of 1.063g/ml. The mixture was then subjected to density gradient ultracentrifugation at 285,000g for 2 hrs, and centrifugation was allowed to stop without use of break. The top 4ml, where the emulsion particles are recovered, and the bottom 5ml of the tubes were collected and assayed for content of lipidized polylysine using the modified Lowry method. Tubes containing lipidized PLL only, and submicron emulsion only were centrifuged along with the mixture samples and used as controls.

Results

Particle size and chemical composition

Fraction	Particle size (nm)	Cholesterol%	Cholesteryl oleate%	Triolein%	Phospholipid%
F1	155	1.910076	6.448696	84.34123	7.3
F2	76	2.123761	6.202347	79.07389	12.6
F3	44	2.706742	5.079775	64.81348	27.4

Degree of lipidized poly lysine association with PSME

The amount of 545μg of m-PLL per 1mg of triolein associated with the 2nd emulsion fraction. This amount was calculated after subtracting the amount of m-PLL from control tube of m-PLL alone that floats to the top and the interference of emulsion turbidity on the analysis of m-PLL associated with the emulsion particles.

Interaction of lipidized poly lysine with lipoprotein-resembling submicron emulsion

Due to the association of m-PLL with submicron emulsion particles the surface charge of the particles was completely reversed. Before addition of m-PLL the emulsion particles had slight negative charge as the case with human LDL too. After the addition of m-PLL which has a positive charge, owing to the free ε-amino groups of lysine, the particles showed movement toward the negative electrode suggesting the charge of the m-PLL associated submicron emulsion is positive. The addition of unmodified polylysine caused the emulsion particles to precipitate immediately. The precipitation can be due to charge neutralization rather than physical incorporation of the polylysine molecules with the surface of the emulsion particles. Lipidization of polylysine appears to be critical for successful association with the emulsion without precipitation. Since DNA is negatively charged, the m-PLL associated emulsion particles has the ability to carry DNA molecules, which can be used for gene delivery. Experiments comparing emulsion compositions without the lipidized polylysine (did not carry DNA molecules) and those with lipidized polylysine (efficiently carried the DNA molecules) confirm these results.

Example 6- Lipidized Avidin

Avidin is a glycoprotein which can be lipidized readily using the above-described methods and associated with the microemulsion system of the present invention, which can then function as a universal station for the attachment of various biotinylated proteins (for example, enzymes, hormones, toxins, antibodies, receptors, etc.) polymers and/or bioactive agents, such as drugs, including those biotinylated proteins which are well known in the art. Lipidized avidin can also be associated with native LDL and therefore allow the binding of biotinylated polymers, protein and drugs to benefit from the LDL pathway for delivery.

The advantage of using this system with a biotinylated antibody include the intact functionality of the antibody (Figure 4). The antibody will be functional since the active site

has not been modified or affected. The approach will also overcome any possible limitations of non-selective modification and disorientation of the antibody in the microemulsion which might occur from direct modification on the antibody

The advantage of using this system with polymers includes the facilitation for targeted gene delivery. Polymers can be lipidized in a similar manner as proteins. A cationic polymer (e.g, polylysine or polyethyleneimine) in a lipidized form which is associated with the microemulsion can be used for gene delivery since cationic polymers are known to complex with DNA through electrical attraction between the negatively charged DNA and positively charged polymers (figure 5).

This artificial LDL system may also behave as native LDL by acquiring apolipoprotein E from the plasma. Apolipoprotein E is known to interact with LDL receptors in a similar manner to Apolipoprotein B 100 but with even higher affinity. Apolipoprotein B 100 is the main protein component present in the native LDL and responsible for interaction with the LDL receptor. Interaction with an LDL receptor initiates endocytosis for the entire LDL particle and therefore the process can be utilized to deliver drug molecules inside the cells.

The following examples illustrate the synthesis of carborane cholesterol compounds (BCH) which may be used in the present invention. The synthesis of the carborane cholesterol compounds according to the present invention is presented in the following experiments. In general, where solvent is used, it is dried and distilled prior to use. Nitrogen is used dry at all times. All other materials are dried and distilled prior to use.

Experiment 7
Synthesis of Carborane acid (1-hydroxycarbonyl-1, 12-dicarba –closo-dodecaborane)

n-BuLi (1.1 ml, 1.66 mmol, 1.6 M in hexane) was slowly added to a stirred solution of ρ-carborane (200 mg, 1.38 mmol) in ether (80ml) in a flask fitted with a reflux condenser at 0 °C . The reaction mixture was warmed to room temperature and refluxed for 3 hr. The reflux condenser was removed and the reaction mixture was cooled to – 78 °C (dry ice/ aceton). Dry ice (CO_2) was added to the reaction mixture under positive flow of nitrogen. The reaction

mixture was allowed to warm to room temperature and excess ether was removed by vacuum. The residue was dissolved in 10ml water and extracted with ether (2x5 ml). The aqueous layer was acidified with HCl solution (5M) to pH 1. The product, p-carborane carboxylic acid, was extracted by ethyl acetate. Proton NMR and ^{13}C NMR have been used to confirm the structure of product. ^{1}H NMR ($CDCl_3$, 400Hz) δ: 1.6-3.2(10H, B-H)(see Fig.2); ^{13}C NMR (400Hz, DMSO-d_6) δ: 166.58, 83.33;

Experiment 8

Synthesis of cholesteryl 1,12-dicarba-closo-dodecaborane 1-carboxylate

ρ-carborane carboxylic acid of 80 mg was placed in 25 ml flask. Thionyl chloride (5 ml) was added and the flask was quickly attached to reflux condenser protected by drying tube. The assembly was mounted in oil bath at 78 °C for 4 hr. Excess SO_2Cl was removed by vacuum. Cholesterol (160mg) was dissolved in methylene chloride (3 ml) (containing pyridine 50 μl). The reaction mixture was stirred under nitrogen for 48 hr. The excess solvent was removed and residue was subject to column chromatography to separate the product from impurity. Proton NMR and ^{13}C NMR have been used to confirm the structure of product. ^{1}H NMR ($CDCl_3$, 400Hz) δ: 1.6-5.1(10H, B-H), 5.34(1H, Chol 6), 4.43(1H,Chol 3); ^{13}C NMR ($CDCl_3$) δ: 61.72(acid 1), 139.24 (chol 5), 122.71 (Chol 6);

Experiment 9

Synthesis of cholesteryl 1,12-dicarba-closo-dodecaborane 1-carboxylate

A mixture containing ρ-carborane carboxylic acid (80mg, 0.4mmol), cholesterol (180mg, 0.4mmol), DCC (dicyclohexylcarbodiimide) (80mg, 0.4mmol), DMAP (4-(dimethylamino) pyridine) (10mg, 0.08mmol) was stirred in 10 ml methylene chloride for 48 hr at room temperature. The reaction mixture was cooled to 0°C and precipitate was filtrated. The filtrate was subject to silica gel column chromatography to purify the product: cholesteryl 1,12-dicarba-closo-dodecaborane 1-carboxylate. Proton NMR and ^{13}C NMR have been used to confirm the structure of product. ^{1}H NMR ($CDCl_3$, 400Hz) δ: 1.6-5.1(10H, B-H), 5.34(1H, Chol 6), 4.43(1H, Chol 3); ^{13}C NMR (400Hz, $CDCl_3$) δ: 61.72 (acid 1), 139.24 (chol 5), 122.71(Chol 6);

Each of the o-carborane cholesterol and m-carborane cholesterol compounds may be synthesized by analogy to the p-carborane cholesterol compound synthesized above in examples 7-9 (example 9 is BCH) substituting o-carborane or m-carborane for p-carborane in the syntheses. The remaining steps would proceed as they are presented, above. In sum the following three compounds are presented: cholesterol 1,12-dicarba-closo-dodecaborane 1-carboxylate, cholesterol 1,2-dicarba-closo-dodecaborane 1-carboxylate, and cholesterol 1,7-dicarba-closo- dodecaborane 1-carboxylate.

It is to be understood by those skilled in the art that the foregoing description and examples are illustrative of practicing the present invention, but are in no way limiting. Variations of the detail presented herein may be made without departing from the spirit and scope of the present invention as defined by the following claims.